

Molecular cloning and sequencing of hemoglobin- β gene of channel catfish, *Ictalurus punctatus* Rafinesque

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Abstract The hemoglobin- β gene of channel catfish, *Ictalurus punctatus*, was cloned and sequenced. Total RNA from head kidneys was isolated, reverse transcribed and amplified. The sequence of the channel catfish hemoglobin- β gene consists of 600 nucleotides. Analysis of the nucleotide sequence reveals one open reading frame and 5'- as well as 3'-untranslated regions. The open reading frame of the sequence potentially encodes 148 amino acids with a calculated molecular mass of 16.3 kDa. The pI and charge at pH 7.0 of the deduced hemoglobin- β protein were 7.28 and 0.47, respectively. Overall, 22 amino acid residues were conserved throughout the sequences, including His64 and His93, the sites for heme-binding. Unlike the counterpart of other common cultured fish such as *Salmo salar*, *Oncorhynchus nerka*, *Oncorhynchus mykiss*, *Cyprinus carpio* and *Ctenopharyngodon idella*, the hemoglobin- β of channel catfish did not have cysteine. The amino acid sequence of channel catfish hemoglobin- β shows 84% homology with that of *Silurus asotus* (both are in the order *Siluriformes*). However, comparison with

those of other fish species shows homology ranging from 53 to 68%. Structural analysis by the 3D-PSSM program displays that channel catfish hemoglobin- β has eight α -helices, A–H.

Keywords Channel catfish · *Ictalurus punctatus* · Hemoglobin- β · Aquaculture

Introduction

Hemoglobin is made up of four polypeptides: two identical α -chain and two identical β -chain polypeptides. The specific characteristic of this protein is its ability to reversibly bind oxygen that is critical for a wide variety of cellular functions. Because hemoglobin has important physiological functions, the hemoglobin gene and its products have been used as a model for studying genetic regulation, protein structure and function, molecular evolution and environmental adaptation (e.g., Bargelloni et al. 1998; Du et al. 2003; Shikama and Matsuoka 2003; Verde et al. 2002).

Fish constitute more than 23,000 species and live in wide range of environments (Helfman et al. 1997) and fish hemoglobins have shown a huge variation in components and oxygen-binding mechanisms (Weber 1982, 1996). For example, Tamburrini et al. (1992) studied the Antarctic fish hemoglobin and found that *Gymnodraco acuticeps* has a single hemoglobin without the Bohr effect, indicating that the role of this

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hemoglobin in oxygen transport is reduced in this extreme environment. On the other hand, Verde et al. (2002) used the hemoglobin of the Arctic fish, *Anarhichas minor* as a model and found that the fish has high hemoglobin multiplicity differing functionally in pH, organophosphate regulation and temperature regulation. However, the hemoglobin gene and functional aspects of hemoglobin from temperate species such as channel catfish have not been described.

Channel catfish production is an important aquacultural industry in the southeastern United States. Its annual output reaches 410 million dollars. In the course of studying catfish physiology, we cloned and sequenced the hemoglobin- β gene of channel catfish, *Ictalurus punctatus*. The deduced amino acid sequence was compared with hemoglobin- β of other species deposited in the GenBank database, and we found that channel catfish did not have cysteine in its hemoglobin- β . Finally, its three-dimensional structure was predicted by the 3D-PSSM program.

Materials and methods

Animals

Two-year-old channel catfish, *Ictalurus punctatus* Rafinesque, NWAC 103 strain were used in this study. The protocol for animal use in experiments was approved by the Institutional Animal Care and Use Committee, Aquatic Animal Health Research Unit, Mid-South Area, Agricultural Research Service, United States Department of Agriculture.

RNA isolation

Total RNA from head kidneys was isolated by using a Tri Reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's instruction. The quality and quantity of the isolated RNA were determined by agarose gel electrophoresis and spectrophotometry, respectively.

Generation of a cDNA library

After total RNA isolation, cDNA was generated by using a GeneRacer kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Briefly,

total RNA was treated with calf intestinal phosphatase to remove the 5'-phosphate, treated with tobacco acid pyrophosphatase to get rid of 5' cap structure and then ligated to a GeneRacer RNA Oligo, 5'-CGA-CUGGAGCACGAGGACACUGACAUGGACUGA-AGGAGUAGAAA-3'. The treated RNA was then reverse transcribed into cDNA and amplified by PCR. Amplification was carried out on a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Waltham, MA) as follows: initial activation of DNA polymerase at 95°C for 5 min, and then denaturation at 95°C (15 sec), annealing at 56°C (1 min) and elongation at 72°C (1.5 min). After 35 cycles, the mixture was incubated at 72°C for 10 min. The PCR products were analyzed by electrophoresis on agarose gels and cloned into the pCR4-TOPO cloning vector (Invitrogen, Carlsbad, CA). The primers used for amplification were: (1) 5'-CGACTGGAGCACGAGGACA CTGA-3', (2) 5'-GCTGTCAACGATACGCTACGT AACG-3' (3) 5'-ATGGTTCATTGGACAGACGCC-3' and (4) 5'-TTAGTGGTACTGCTTCCCAG-3'.

DNA sequencing

The DNA sequencing reactions were carried out at the Auburn University Genomics and Sequencing Laboratory with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Gene analysis

The sequences of nucleotides and deduced amino acids were aligned by using ClustalX (v.1.83) (Thompson et al. 1997). Tree figures were generated by using the TreeView program (1.5.2) (Page 1996). The phylogenetic and molecular evolutionary analyses were conducted using the MEGA (v. 2.1) program (Kumar et al. 2001).

Three-dimensional model

The web-based program of three-dimensional position-specific scoring matrix (3D-PSSM) (Kelley et al. 2000) was used to predict secondary structure and create a 3D model of the channel catfish hemoglobin- β . The principle of this method is to map the coordinates of the template structure and the query sequence residues aligned to them. If a confident hit is found, the model is further enhanced by the

addition of side-chains using the SCWRL algorithm (Kelley et al. 2000).

Accession No.

The hemoglobin- β cDNA sequence was deposited in the GenBank database and its Accession No. is AY462104.

Results and discussion

Cloning and sequencing of channel catfish hemoglobin- β gene

Using the BLAST algorithm program (Altschul et al. 1997), the complete cDNA sequence consisting of

600 nucleotides of the channel catfish was found to correspond to the hemoglobin- β gene. Analysis of the nucleotide sequence revealed one open reading frame and 5'- as well as 3'-untranslated regions (Fig. 1). As in mammals, the 5'-untranslated regions had 61 bases. The 3'-untranslated region had 92 bases in length with a consensus polyadenylation signal. The open reading frame potentially encoded 148 amino acids with a calculated molecular mass of approximate 16.3 kDa. In addition, the predicted hemoglobin- β had two potential sites for glycosylation (residues 48 and 103) and three potential sites for myristoylation (residues 26, 47 and 71), respectively. It is known that fish hemoglobin- β protein can be divided into anodic and cathodic types based on its electrophoretical properties (Weber 1996). We further analyzed the deduced hemoglobin- β protein

Fig. 1 Nucleotide sequence (lowercase) and predicted amino acid sequence (uppercase in the one-letter amino acid code) of channel catfish hemoglobin- β . Potential *N*-glycosylation sites at residues 48 and 103 are indicated by bold and italic. Potential myristoylation sites at residues 26, 47 and 71 are indicated by underline. *, stop codon

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1      aaaaagattcgttcagaacaggtttgctccaaattaaaagaattcatt

          M   V   H   W   T   D   A   E   R   H   I   I   A
51     aagacatcaatatggttcattggacagacgcgcagcgcatacatcgct

          D   L   W   G   K   I   N   H   D   E   I   G   G   Q   A   L   A
101    gaccttggggaaagatcaaccatgacgaaatcgaggacaggccctggc

          R   L   L   I   V   Y   P   W   T   Q   R   Y   F   S   S   F
151    cagacttctgatttgttatccatggacccagaggtacttcttcctttg

          G   N   L   S   N   A   A   A   I   I   G   N   P   K   V   A   A
201    gtaatctatccaatgccgcagccatcattggaaatcccaagggttgctgcc

          H   G   K   V   V   L   G   G   L   T   K   A   V   Q   N   L   D
251    catggaaaggtggtccttggctgaccaaagctgtgcaaaatttggaa

          N   I   K   G   I   Y   T   Q   L   S   T   L   H   S   E   K
301    caacattaaggcatctacactcagctgagtacgcttactctgaaaaac

          L   H   V   D   P   S   N   F   T   L   L   G   D   T   F   T   V
351    tgcacgtggatcccagcaacttcacgcttgggtgacaccttaccgtaa

          T   L   A   A   N   F   G   P   S   V   F   T   P   E   V   H   E
401    actctggccgcaatttcggaccctcagtcgttgcacccctgaagtgcacga

          T   W   Q   K   F   L   N   V   V   V   A   A   L   G   K   Q
451    gacttggcagaagttcctgaatgtcgctgtggctgtctggaaagcagt

          Y   H   *
501    accactaaacacgcataatataaaagatgtgcaggctttactctgt

          gactagaacatgaaataaaacgttaaacagcaaaaaaaaaaaaaaaaaaa
551

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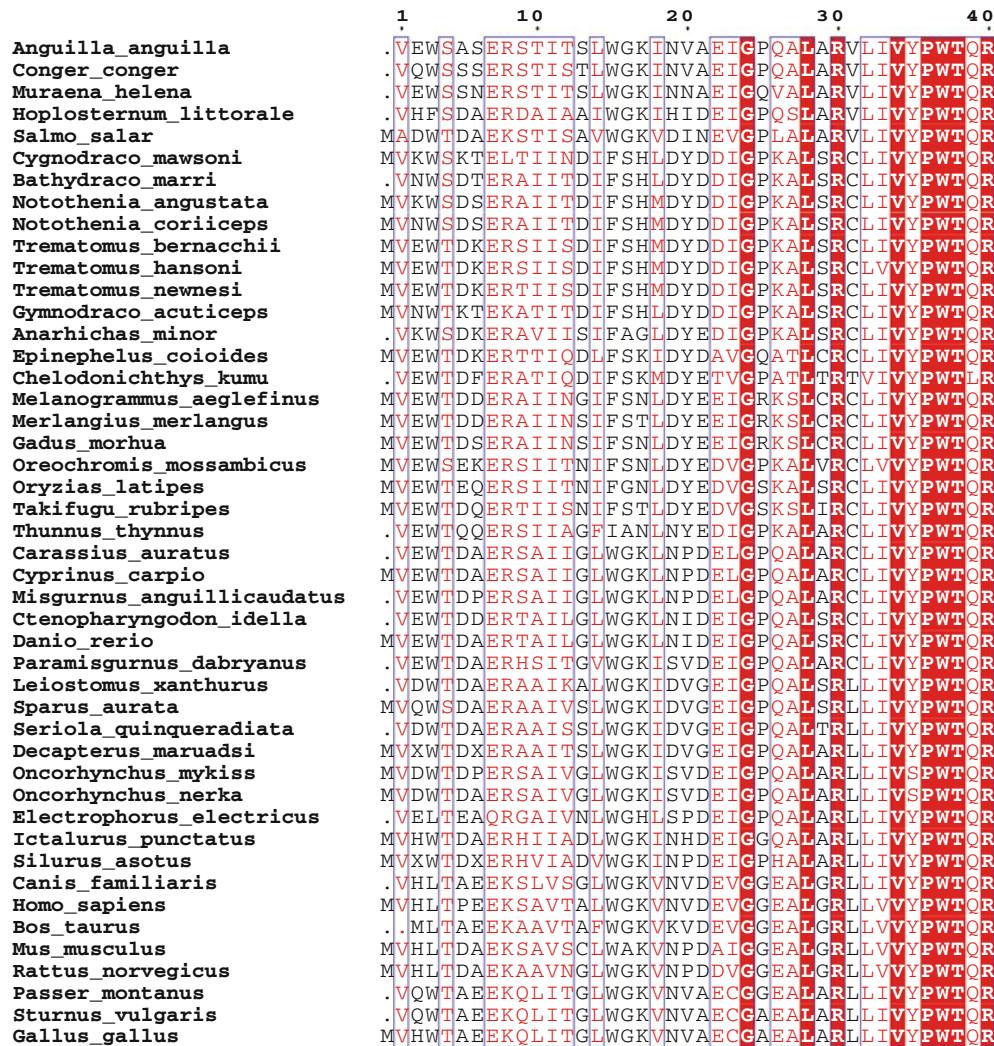
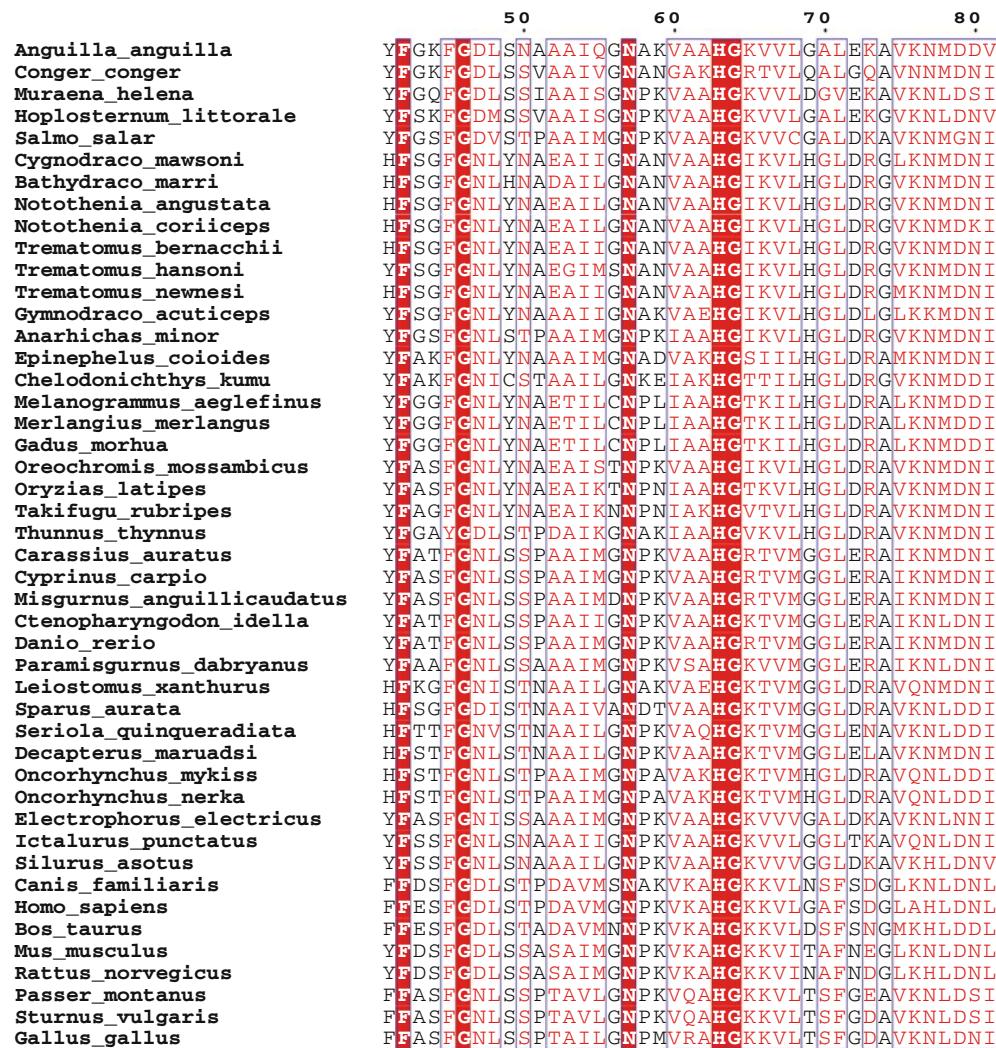


Fig. 2 The deduced amino acid sequence of channel catfish (*Ictalurus punctatus*) hemoglobin- β aligned with the amino acid sequences of other fish hemoglobin- β deposited in public domains. Sequences were aligned by using the ClustalX program (Thompson et al. 1997). To optimize maximal alignment, gaps were introduced in the sequences indicated as dots. The conserved amino acids are indicated in white letters with red background. The fish scientific names of each sequence are on the left. *Anarhichas minor*, P83272; *Anguilla anguilla*, P80946 (Fago et al. 1997); *Bathydraco marri*, B56898 (Caruso et al. 1992); *Bos Taurus*, NM_173917; *Canis familiaris*, P02056 (Brimhall et al. 1997); *Carassius auratus*, P02140 (Rodewald and Braunitzer 1984); *Chelodonichthys kumu*, P80271; *Conger conger*, P83478 (Pellegrini et al. 2003); *Ctenopharyngodon idella*, AAM93253; *Cygnodraco mawsoni*, AAC41384; *Cyprinus carpio*, BAA20516; *Danio rerio*, NP_571096; *Decapterus maruadsi*, O13164 (Suzuki and Nishikawa 1996); *Electrophorus electricus*, P14521 (Huber and Braunitzer 1989); *Epinephelus coioides*, AAK38736; *Gadus morhua*, O13077; *Gallus gallus*, P02112 (Richards et al. 1979); *Gymnodraco acuticeps*, AAC41386; *Homo sapiens*, NM_000518 (Marotta et al. 1976); *Hoplosternum littorale*, P82316 (Weber et al. 2000); *Ictalurus punctatus*, AY462104; *Leiostomus xanthurus*, P56251; *Melanogrammus aeglefinus*, O09232; *Merlangius merlangus*, O13078; *Misgurnus anguillicaudatus*, AAM93260; *Muraena helena*, Q7LZC (Pellegrini et al. 1995); *Mus musculus*, BC032264 (Strausberg et al. 2002); *Notothenia angustata*, AAF36818; *Notothenia coriiceps*, I51012 (Lau et al. 2001); *Oncorhynchus mykiss*, BAA11632; *Oncorhynchus nerka*, Q98TS0; *Oreochromis mossambicus*, AAP13060; *Oryzias latipes*, BAC06483 (Miyayama et al. 2002); *Paramisgurnus dabryanus*, AAM93251; *Passer montanus*, P07406 (Schneegansse et al. 1985); *Rattus norvegicus*, BC058448; *Salmo salar*, CAA65950 (McMorrow et al. 1996); *Seriola quinqueradiata*, BAA86220 (Okamoto et al. 2001); *Silurus asotus*, O13163; *Sparus aurata*, CAB83257; *Sturnus vulgaris*, P02126 (Oberthur and Braunitzer 1984); *Takifugu rubripes*, AAO61493; *Thunnus thynnus*, P11749 (Rodewald et al. 1987); *Trematomus bernacchii*, P80044; *Trematomus hansonii*, O93351; *Trematomus newnesi*, O93349

sapiens, NM_000518 (Marotta et al. 1976); *Hoplosternum littorale*, P82316 (Weber et al. 2000); *Ictalurus punctatus*, AY462104; *Leiostomus xanthurus*, P56251; *Melanogrammus aeglefinus*, O09232; *Merlangius merlangus*, O13078; *Misgurnus anguillicaudatus*, AAM93260; *Muraena helena*, Q7LZC (Pellegrini et al. 1995); *Mus musculus*, BC032264 (Strausberg et al. 2002); *Notothenia angustata*, AAF36818; *Notothenia coriiceps*, I51012 (Lau et al. 2001); *Oncorhynchus mykiss*, BAA11632; *Oncorhynchus nerka*, Q98TS0; *Oreochromis mossambicus*, AAP13060; *Oryzias latipes*, BAC06483 (Miyayama et al. 2002); *Paramisgurnus dabryanus*, AAM93251; *Passer montanus*, P07406 (Schneegansse et al. 1985); *Rattus norvegicus*, BC058448; *Salmo salar*, CAA65950 (McMorrow et al. 1996); *Seriola quinqueradiata*, BAA86220 (Okamoto et al. 2001); *Silurus asotus*, O13163; *Sparus aurata*, CAB83257; *Sturnus vulgaris*, P02126 (Oberthur and Braunitzer 1984); *Takifugu rubripes*, AAO61493; *Thunnus thynnus*, P11749 (Rodewald et al. 1987); *Trematomus bernacchii*, P80044; *Trematomus hansonii*, O93351; *Trematomus newnesi*, O93349

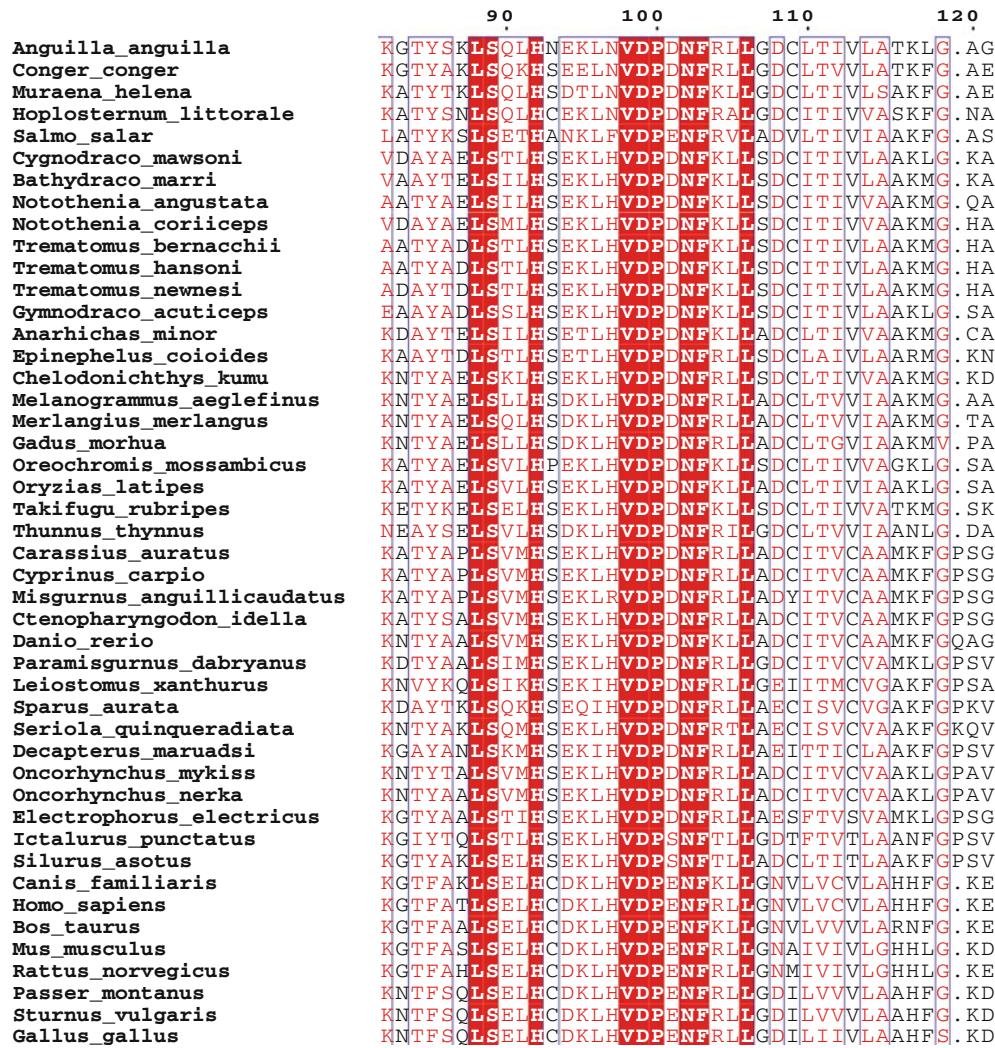
**Fig. 2** continued

and found that its pI and charge at pH 7.0 were 7.28 and 0.47, respectively, indicating that the deduced channel catfish hemoglobin- β protein is anodic. Several clones were sequenced and the same results were obtained. Similar expressed sequence tags for channel catfish hemoglobin- β were deposited in the GenBank's EST database by Z.J. Liu (<http://www.ncbi.nlm.nih.gov/dbEST/index.html>).

Alignment of channel catfish hemoglobin- β with other vertebrate hemoglobin- β amino acid sequences

To determine the relatedness of the predicted channel catfish hemoglobin- β with known hemo-

globin- β amino acid sequences deposited in GenBank, the ClustalX program (Thompson et al. 1997) was used to align hemoglobin- β amino acid sequences (Fig. 2). Overall, 22 amino acid residues were conserved throughout the sequences. These are (positions in reference to channel catfish hemoglobin- β sequence) Gly25, Arg31, Val35, Pro37-Trp38-Thr39, Arg41, Phe43, Gly47, Asn58, His64-Gly65, Leu89-Ser90, His93, Val99-Asp100-Pro101, Asn103-Phe104, Leu107 and Lys134. Among them His64 and His93 were the sites for heme-binding. Also, the residue His147 that is regarded for accounting for over 50% of the Bohr effect (Shih et al. 1993) is conserved in the channel catfish hemoglobin- β . His93 and Tyr36 were conserved;

**Fig. 2** continued

these are considered to have an ancient origin (Freitas et al. 2004). Common cultured fish such as *Salmo_salar* (Wagner et al. 1994), *Oncorhynchus_nerka* (Accession No.: Q98TS0), *Oncorhynchus_mykiss* (Accession No.: BAA11632), *Cyprinus_carpio* (Miyata and Aoki 1997) and *Ctenopharyngodon_idella* (Accession No.: AAM93253) have cysteines in their hemoglobin- β molecules. However, channel catfish hemoglobin- β did not have cysteine. The pair-wise comparison of amino acid sequences showed the channel catfish hemoglobin- β was highly conserved (84%) with *Silurus_asotus* (Amur catfish) but less so (53–68%) between different fish families and 54% with that of *Homo_sapiens* (human).

Phylogenetic construction

To determine evolutionary relatedness among vertebrate hemoglobin- β , the phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar et al. 2001) based on the ClustalX alignment. The tree constructed by the Neighbor-Joining method demonstrated that avian (Accession Nos.: P07406, P02126 and P02112) and mammalian (Accession Nos.: BC032264, BC058448, NM_173917, NM_000518 and P02056) hemoglobin- β formed two well-supported clades (Fig. 3). On the other hand, because fish are a heterogeneous group of more than 20,000 species, they did not form a single well-supported clade. The tree also shows that the

	1 3 0	1 4 0
Anguilla_anguilla	FP AEI QAVWOKFVAVVVSALSKQY	F . . .
Conger_conger	FP PEVQAVWOKFVAVVVSALSROY	F . . .
Muraena_helena	FT PAVQAVWOKFLSCVIALSRQY	F . . .
Hoplosternum_littorale	FT PELQNAWOKFLSVVAAALSSRY	F . . .
Salmo_salar	FT PEI QATWOKFMKVVAAMGSRY	F . . .
Cygnodraco_mawsoni	FTAETQAAFKFLAVVVSALGKQYH
Bathydracc_marri	FTAETQAAFKFLAVVVSALGKQYH
Notothenia_angustata	FT PETQAAVOKFLAVVVSALGKQYH
Notothenia_coriiceps	FT PEI QGAFOKFLAVVVSALGKQYH
Trematomus_bernacchii	FTAETQGAFOKFLAVVVSALGKQYH
Trematomus_hansoni	FTAETQGAFOKFLAVVVSALGKQYH
Trematomus_newnesi	FTAETQGAFOKFLAAVVSALGKQYH
Gymnodraco_acuticeps	FTAETQATFOKFLGAVMSALGKQYH
Anarhichas_minor	FTPDTQLAFOKFLAVVVSALGKQYC
Epinephelus_coioides	FT PEI QATIOKFMADVVSALGRQYH
Chelodonichthys_kumu	FT GEVQAAFKFLSVVVNSLGRQYH
Melanogrammus_aeglefinus	FT VDTQVAWOKFLAVVVSALGRQYH
Merlangius_merlangus	FT VETQVAWOKFLAVVVSALGRQYH
Gadus_morhua	FT VDTQVGWOKFRSFVVSALGREYH
Oreochromis_mossambicus	FT PEVQATFOKFLAVVVSALGKQ
Oryzias_latipes	FSPEI QATFOKFLAVVVSALGRQYH
Takifugu_rubripes	FT PEI QATFOKFLAVVVSALGRQYH
Thunnus_thynnus	FT VETQCAFOKFLAVVVFALGRKYH
Carassius_auratus	FN ADVQEAWOKFLSVVVSALCRQYH
Cyprinus_carpio	FN ADVQEAWOKFLCVVVVSALCRQYH
Misgurnus_anguillicaudatus	FS ANVQEAWOKFLSVVVSALCRQYH
Ctenopharyngodon_idella	FN ADVQEAWOKFLSVVVSALCRQYH
Danio rerio	FN ADI QEAWOKFLAVVVSALCRQYH
Paramisgurnus_dabryanus	FTP DVHEAWOKFLSVVVSALCRQYH
Leiostomus_xanthurus	FT PEI HEAWOKFLAVVVSALGRQYH
Sparus_aurata	LN ADAQEAWOKFLAVVVARILANSTTEALE
Seriola_quinqueradiata	FT ADVQEAWOKFLSVVVSALGRQYH
Decapterus_maruadsi	FT PDFQEAWOKFENAVVVSALGRQYH
Oncorhynchus_mykiss	FS ADTQEAFOKFLAVVVSALGRQYH
Oncorhynchus_nerka	FN ADTQEAFOKFLAVVVSALGRQYH
Electrophorus_electricus	FNAETQHALAKFLAEVVSALGKQYH
Ictalurus_punctatus	FT PEVHETWOKFLNVVVAALGKQYH
Silurus_asotus	FT PEVHEVWOKFLNVAVAALGKQYH
Canis_familiaris	FT PQVQAAAYOKVVAAGVANALAHKYH
Homo_sapiens	FT PPVQAAAYOKVVAAGVANALAHKYH
Bos_taurus	FT PVLQADFOKVVAAGVANALAHRYH
Mus_musculus	FT PAAQAAFOKVVAAGVATAIAHKYH
Rattus_norvegicus	FT PCAQAAFOKVVAAGVASALAHKYH
Passer_montanus	FT PDCQAAWOKLVRVVAHALARKYH
Sturnus_vulgaris	FT PDCQAAWOKLVRVVAHALARKYH
Gallus_gallus	FT PECQAAWOKLVRVVAHALARKYH

Fig. 2 continued

channel catfish hemoglobin- β was close to that of *Silurus asotus* (Accession No.: O13163) (Fig. 3), suggesting that the phylogeny based on the classical taxonomy. Results from the maximum parsimony method yielded the same conclusion.

Molecular modeling

To determine the three-dimensional structure of the channel catfish hemoglobin- β , we submitted the amino acid sequence of the hemoglobin- β via Internet to the 3D-PSSM Web Server (v.2.6.0) for comparison against the templates deposited in the protein structure

databases. We found that the residues of the channel catfish hemoglobin- β was 69% identical to that of teleost fish, *Leiostomus xanthurus*. This protein was an alpha protein, belonging to the globin super-family. Like other hemoglobin- β , the secondary structure of the channel catfish hemoglobin- β had eight α -helices: A, 6–15; B, 21–24; C, 26–30; D, 40–43; E, 52–56; F, 59–95; G, 101–119 and H, 126–144 (Amino acids numbering after the *Ictalurus punctatus* sequence). The three-dimensional molecular model showing heme group was similar to the template of *Leiostomus xanthurus* hemoglobin- β (database i.d. d1spgb) (Fig. 4), reflecting that although the primary amino

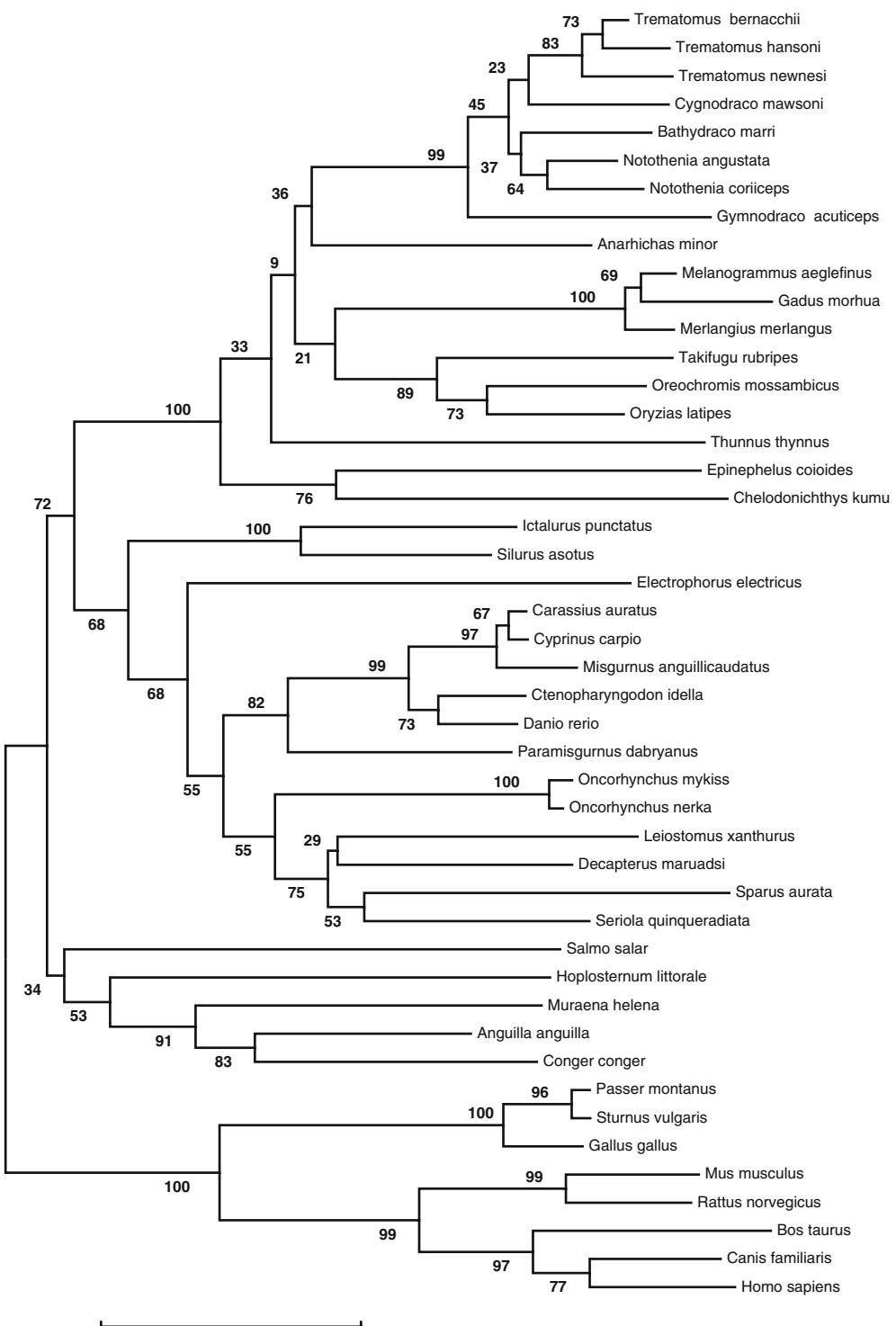


Fig. 3 Molecular phylogenetic relationships of hemoglobin- β amino acids. Sequences from Fig. 2 were used to generate the phylogenetic tree by bootstrap analysis (500 replications) in the

MEGA2 phylogenetic analysis program (Kumar et al. 2001). The numbers on the tree are bootstrap P values

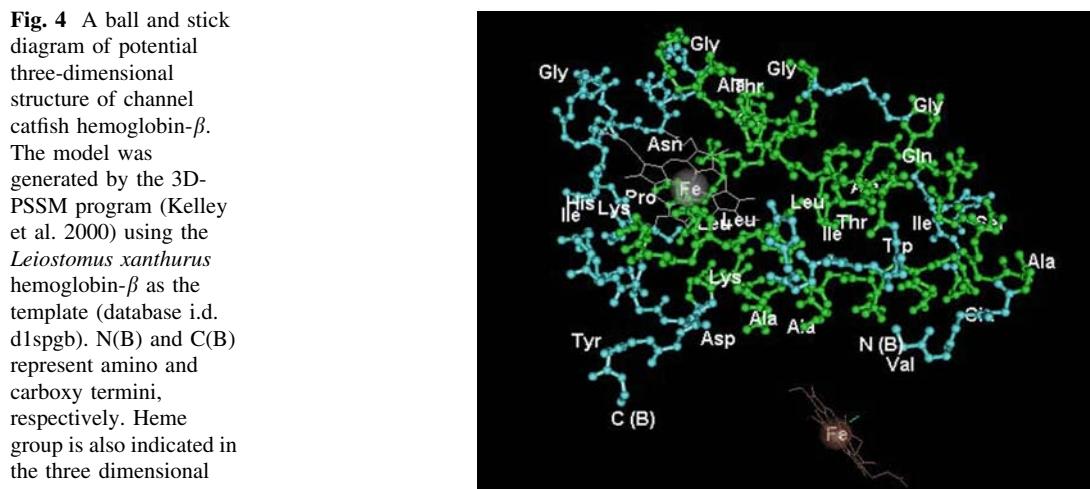
Fig. 4 A ball and stick diagram of potential three-dimensional structure of channel catfish hemoglobin- β . The model was generated by the 3D-PSSM program (Kelley et al. 2000) using the *Leiostomus xanthurus* hemoglobin- β as the template (database i.d. d1spgb). N(B) and C(B) represent amino and carboxy termini, respectively. Heme group is also indicated in the three dimensional structure

acid sequences are divergent, the three-dimensional structure of the hemoglobin- β protein among species is conserved during the evolution.

In summary, the sequence of the channel catfish hemoglobin- β gene consists of 600 nucleotides, potentially encoding 148 amino acids with a calculated molecular mass of 16.3 kDa. Overall, 22 amino acid residues were conserved throughout the sequences, including His64 and His93 that are the sites for heme-binding. Its hemoglobin- β molecules did not have cysteine. The amino acid sequence of channel catfish hemoglobin- β shows 84% homology with that of *Silurus asotus* (both are in the order of *Siluriformes*). However, comparison with those of other fish species shows homology ranging from 53 to 68%. The structural analysis by the 3D-PSSM program displays that channel catfish hemoglobin- β has eight α -helices, A–H.

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